Early Journal Content on JSTOR, Free to Anyone in the World

This article is one of nearly 500,000 scholarly works digitized and made freely available to everyone in the world by JSTOR.

Known as the Early Journal Content, this set of works include research articles, news, letters, and other writings published in more than 200 of the oldest leading academic journals. The works date from the mid-seventeenth to the early twentieth centuries.

We encourage people to read and share the Early Journal Content openly and to tell others that this resource exists. People may post this content online or redistribute in any way for non-commercial purposes.

Read more about Early Journal Content at http://about.jstor.org/participate-jstor/individuals/early-journal-content.

JSTOR is a digital library of academic journals, books, and primary source objects. JSTOR helps people discover, use, and build upon a wide range of content through a powerful research and teaching platform, and preserves this content for future generations. JSTOR is part of ITHAKA, a not-for-profit organization that also includes Ithaka S+R and Portico. For more information about JSTOR, please contact support@jstor.org.

VOLUME XXXIX NUMBER 5

BOTANICAL GAZETTE

MAY, 1905

ON PROTEOLYTIC ENZYMES. I.

ARTHUR L. DEAN.

NUMEROUS investigators have studied the proteolytic enzymes in plants and their work appears to have been carried on from two different points of view. The physiological chemists working with vegetable proteases have made careful studies of the products and nature of the action of enzymes of marked activitity. Of such work, the investigations carried on by CHITTENDEN and his pupils on bromelin are excellent examples. In studies of this kind the enzyme is the important thing, the rôle of the enzyme in the physiology of the plant is secondary. On the other hand, the studies of plant physiologists have been directed less to working out the nature of the enzymes and more to locating them and to discovering their function in the life of the plant. Of such researches the intensive studies of BUTKEWITSCH on germinating seeds, and the extensive ones of VINES on all sorts of plant tissues, are good illustrations.

Looking at the subject from the point of view of plant physiology, we may conceive of proteolytic enzymes as being digestive, *i. e.*, acting on stores of proteid, as in germinating seeds; or metabolic, *i. e.*, acting on the proteids in the life of the vegetable cells. The idea of enzymes digesting the reserve proteids was the first to occur to physiologists. Acting on the hypothesis of the existence of such enzymes, numerous investigators have examined germinating seeds, using a variety of methods, and obtaining results which have not always been in harmony with the hypothesis or with each other.

The view that wherever we find proteids in an organism, there we should always find a proteolytic enzyme, is not a new one; the experimental facts to support such a conception have been slow in coming. Manifestly the first step in proving this assumption is to show that proteolytic enzymes are present in all parts of all living plants, or to demonstrate their presence in a sufficient number of places to warrant the conclusion that they are universally present. Such extensive researches have not been undertaken until the last few years.

I shall briefly review the results of the various investigations, taking first the studies on the digestive enzymes, as I have called them, and then those on the metabolic enzymes.

GORUP-BESANEZ[1] appears to have been the first to examine seeds for proteolytic enzymes. Using the Wittich glycerin method he obtained protease preparations from the resting seeds of hemp, flax, and vetch, and from the germinating barley; he failed to find enzymes in ungerminated barley, lupin seeds, *Secale cornutum*, pine, and corn, and in seedlings of almonds and beans.

VAN DER HARST[2] found that a glycerin extract of the cotyledons of germinating beans would digest fibrin.

The first careful study of a seed proteolytic enzyme was that of J. REYNOLDS GREEN[3] who investigated the germinating seeds of *Lupinus hirsutus*. GREEN'S results showed conclusively that these plants contain an active enzyme, capable of attacking fibrin, as well as the proteids of the seeds themselves. The enzyme was not present in the resting seeds. Later GREEN discovered a similar enzyme in the seedlings of the castor oil bean.[4]

NEUMEISTER[5] undertook the study of a number of seeds and seedlings, using a method different from that of any previous investigator in this field. Pieces of fibrin were soaked in the watery extracts from the tissues to be tested; subsequently the fibrin was transferred to a weakly acid solution and allowed to digest. In case a proteolytic enzyme was present it was taken up by the fibrin, and in the acid medium proteolysis of the fibrin took place. The value of the negative results by this method has been called in question by FRANKFURT.[6] NEUMEISTER'S results may be summarized as follows:

---

[1] GORUP-BESANEZ, Ber. Deutsch. Chem. Gesells. **7**:1478. 1874; **8**:1510. 1875; and **9**:673. 1876.

[2] VAN DER HARST, Moonblad voor Naturwetensch. 1867, Sept. 20.

[3] GREEN, J. REYNOLDS, Phil. Trans. **178**:(B) 39. 1887.

[4] GREEN, J. REYNOLDS, Proc. Roy. Soc. **48**:370. 1890.

[5] NEUMEISTER, Zeitschr. für Biologie **30**:447. 1894.

POSITIVE RESULTS	NEGATIVE RESULTS
Barley seedlings—sprouts 3^{cm} long.	Barley seeds.
Barley seedlings—sprouts 5^{cm} long.	Barley seedlings—sprouts 0.5 to 1^{cm}.
Barley seedlings—sprouts $15–20^{\mathrm{cm}}$ long.	
Poppy seedlings.	Poppy seeds.
Beet seedlings.	Beet seeds.
Corn seedlings—sprouts 10^{cm} long.	Corn seeds.
Corn seedlings—sprouts 16^{cm} long.	Lupin seeds.
Wheat seedlings—sprouts 17^{cm} long.	Lupin seedlings—sprouts 3^{cm}.
	Lupin seedlings—sprouts 12^{cm}.
	Lupin seedlings—sprouts 20^{cm}.
	Vicia seeds.
	Vicia seedlings.
	Pea seeds.
	Pea seedlings.
	Rye seeds.
	Rye seedlings.
	Oat seeds.
	Oat seedlings.

A number of different investigators have worked on malt; the evidence shows that the germinating barley contains a proteolytic enzyme. WEISS[7] thinks there are two, a peptic and a tryptic enzyme, a view which is supported by FERNBACH and HUBERT,[8] but questioned by VINES.

In the course of the extensive studies of FERMI and BUSCALIONI[9] on proteases in plants, they tested a number of seeds and found that the ripe seeds of several plants contain an enzyme capable of liquefying gelatin. They likewise found that the germinating seeds of some plants contained such enzymes, but in other cases no evidence of their presence could be found.

BUTKEWITSCH[10] made a careful study of the germinating seeds of two lupins, *L. angustifolius* and *L. luteus*, and a somewhat shorter examination of *Ricinus major* and *Vicia Faba*. His experiments show that the proteids in the sprouting seeds of all these species will undergo autolysis, giving rise to products not precipitable by phosphotungstic acid. From *L. luteus* he made a proteid preparation by the Wittich method (solution in glycerin and precipitation by alcohol) which acted on "conglutin" with the formation of leucin and tyrosin, but not of asparagin.

---

[6] FRANKFURT, Landw. Versuchsstat. **47**:466. 1896.

[7] WEISS, Zeitschr. für Physiol. Chem. **31**:79.

[8] FERNBACH and HUBERT, Compt. Rend. **130**:1783.

[9] FERMI and BUSCALIONI, Centralbl. Bact., etc. II. **5**:24. 1899.

[10] BUTKEWITSCH, Zeitschr. Physiol.-Chem. **32**:1. 1901.

VINES[11] found some difficulty in getting a formation of tryptophan in the autolysis of germinating seeds of *Vicia Faba;* the reaction was readily obtained, however, if Witte peptone was added to the mixture and digestion allowed to proceed. He found that the embryo of the wheat contains an enzyme which readily attacks Witte peptone and apparently has some slight action on some of the proteids of the embryo.

As stated above, the first step in demonstrating that proteases are universally at work where proteids occur, is to show that all living plant tissues contain proteolytic enzymes. Investigations of this sort have been undertaken but recently. The older researches, which did not have this purpose, did tend to show that active proteases occur in places other than germinating seeds. The papain of the pawpaw fruit, the bromelin of the pineapple juice, the cradeïn of the latex and juice of the fruit of the fig, and the protease of the Kachree gourd and of *Anagallis arvensis* have been known for some time, but these plants were looked upon as exceptional.

FERMI and BUSCALIONI[12] were the first to make a systematic examination of a large number of plant tissues. Their method consisted in placing the objects to be tested on gelatin containing phenol as an antiseptic. If the gelatin was liquefied by the tissue or tissue extract, a protease was judged to be present. The positive results of this method are valuable; negative results cannot be taken as conclusive evidence that no proteolytic enzyme is present, since all proteases do not attack gelatin, and phenol is said to have an inhibitory influence on the action of some enzymes. These investigators succeeded in showing that proteases occur in a great diversity of plants and of plant parts. Their negative results are sufficiently numerous to cause some doubt in respect to the universal occurrence of proteases in living tissues, and the positive results were obtained with such a diversity of tissues that they do not lead to any definite hypothesis of the physiological rôle of these enzymes. In view of the evidence of VINES' researches, it appears probable that a more thorough study of the tissues examined by FERMI and BUSCALIONI would show that proteases are present there which cannot act under the conditions of their experiments. Their positive results support the theory of the universal distribution of proteolytic enzymes, their negative results do not disprove it.

---

[11] VINES, Annals of Botany **16**:12. 1902.

[12] FERMI and BUSCALIONI, Centralbl. Bact., etc., II. **5**:24. 1899.

Finally we come to a consideration of the results of VINES.[13] He believes that all vegetable proteases are proteolytic in distinction from peptonizing, *i. e.*, that they change the proteids which they attack into their ultimate cleavage products, amido-acids, hexon bases, etc., and do not stop, like pepsin, with the albumoses and peptones in preponderance. Acting on this well-justified assumption, he expects to find tryptophan as the result of the action of vegetable proteases, and uses as a test for proteolysis the progressive formation of tryptophan as shown by the color reaction with chlorine or bromine water. He uses the method of autolysis, allowing the enzymes of a tissue to digest the proteids of that tissue and from time to time tests samples of the mixture for tryptophan. He frequently adds other proteids to the digestion, especially Witte peptone. The only objection to the method is that some proteids do not contain the tryptophan group, or only a small amount of it. A negative result by VINES' method with a tissue containing such a proteid would not mean much. This probably explains the differences between the results obtained by VINES and BUTKEWITSCH in their examinations of the germinating seeds of *Vicia Faba*, since OSBORNE and HARRIS[14] have shown that the proteids of this seed give but a faint Adamkiewicz reaction, a reaction which HOPKINS and COLE[15] have shown to be due to the presence of the tryptophan group in the proteid molecule. By using Witte peptone, VINES obtains evidence of proteolysis in cases where the tissue contains

[13] VINES, Annals of Botany **16**:1. 1902; **17**:237 and 597. 1903; and **18**:289. 1904.

[14] OSBORNE and HARRIS, Jour. Am. Chem. Soc. **25**:853. 1903.

[15] HOPKINS and COLE, Jour. Physiology **27**:419. 1901.

no peptonizing enzyme, but does have an enzyme which, like erepsin of the animal body, attacks albumoses and peptones. In cases where all the trials were made with the addition of Witte peptone, it is impossible to say whether there is an enzyme present of the trypsin type (tryptase), the erepsin type (ereptase), or both. In cases where a positive result was obtained both with or without the addition of Witte peptone, a tryptase is present if, as is probable, the tissue contains no marked quantity of albumoses or peptones; an ereptase may also be present and in the table its presence is indicated as questionable. When no tryptophan reaction is given on autolysis, except after the addition of a proteose, I have assumed that no tryptase was present, an assumption which, as pointed out above, is not entirely justified because certain proteids give no reaction for the tryptophan radical (see table).

This table records but three failures to obtain evidence of a protease, the pulp of the apple, the juice of the orange, and the sap of the bleeding birch tree (*Betula alba*), which last is not a cell extract. The two failures, the apple and the orange juice, are scarcely sufficient to prevent the acceptance of these results as evidence of the universal occurrence of proteases in living plant tissues.

No mention has been made of the proteolytic enzymes of the insectivorous plants and the bacteria and lower fungi. Some of the latter are doubtless endo-enzymes and belong to the group which I have termed metabolic enzymes. The others are apparently secretions designed to render food materials available and should be classed with the digestive enzymes.

The results of VINES tend to show that there are enzymes of the erepsin type widely distributed in plants, yet the evidence is not wholly conclusive and he has made no attempt to isolate such enzymes. DELEZENNE and MOUTON[16] made extracts from several fleshy fungi, *Amanita muscaria*, *Amanita citrina*, *Hypholoma fasciculare*, and *Psalliota* (*Agaricus*) *campestris*, which were judged to contain an ereptase since they digested proteoses but not fibrin. VINES finds, however, that *Agaricus campestris*, which DELEZENNE and MOUTON found to yield an especially active ereptase, is capable of autolysis and will digest fibrin if the fibrin is not boiled. The unboiled fibrin

---

[16] DELEZENNE and MOUTON, Compt. Rend., April 9, 1903.

Tissue	Tryptase	Ereptase	Ereptase or Tryptase or both
Milk of *Cocos nucifera*	—	+	. .
Pilei of *Agaricus campestris*	+	+ ?	. .
Unripe seeds of *Pisum sativum*	+	+ ?	. .
Fruit of *Cucumis melo*	+	+ ?	. .
Fruit of *Cucumis sativus*	+	+ ?	. .
Fruit of *Cucurbita pepo-ovifera*	. .	. .	+
Fruit of *Ecballium Elaterium*	—	+	. .
Fruit of *Musa sapientum*	+	+ ?	. .
Fruit of *Lycoperiscum esculentum*	+	+ ?	. .
Fruit of *Pyrus malus* (pulp)	—	—	—
Fruit of *Pyrus malus* (skin)	—	+	. .
Fruit of *Pyrus communis*	—	+	. .
Fruit of *Citrus aurantium* (juice)	—	—	—
Fruit of *Citrus aurantium* (peel)	?	+	. .
Fruit of *Vitis vinifera*	. .	. .	+
Sap of *Betula alba*	—	—	—
Shoots of *Crambe maritima*	— ?	+	. .
Yeast	+	+ ?	. .
Latex of *Euphorbia Characias*	—	+	. .
Latex of *Lactuca sativa*	+ ?	+	. .
Stems of *Dahlia variabilis*	—	+	. .
Stems of *Cucurbita pepo-ovifera*	+	+ ?	. .
Stems of *Mirabilis Jalapa*	+	+ ?	. .
Stems of *Helianthus tuberosus*	+	+ ?	. .
Stems of *Cuscuta*	—	+	. .
Leaves of *Spinacia oleracea*	+	+ ?	. .
Leaves of *Dahlia variabilis*	+	+ ?	. .
Leaves of *Tropaeolum majus*	+	+ ?	. .
Leaves of six other species of plants	+	+ ?	. .
Leaves of *Holcus mollis* and three other species of plants	. .	. .	+
Bulbs of *Tulipa* and two other plants	+	+ ?	. .
Tubers of *Solanum tuberosum*	+	......	......
Tubers of *Helianthus tuberosus*	+	+ ?	. .
Roots of *Dahlia variabilis*	+	+ ?	. .
Roots of *Brassica Rapa* and seven other plants	—	+	. .

which VINES used would not digest itself and he believes its solution was not due to any enzyme which the fibrin itself contained. VINES agrees with DELEZENNE and MOUTON in believing that this fungus contains an enzyme of the erepsin type.

EXPERIMENTAL DATA

In undertaking the study of the proteolytic enzymes I have had two ends in view. In the first place it was thought desirable to test a number of different plant tissues in order to confirm, if possible, the results of VINES. The second and more important part of the investigation was to make a careful study of the enzymes in some one or two plants, tracing their occurrence and studying their nature throughout the life history of the plant. In this way it was hoped that some results might be obtained which would throw light on the nature and functions of the proteases in the life of plants. For this study the common field bean, *Phaseolus vulgaris*, which has a large proteid content in its seeds and shows marked vigor and rapidity in its growth, was selected. The work on the enzymes of Phaseolus has proved so absorbing that scant attention has been paid to the testing of various plants. The few positive results which have been obtained will be set down at this time.

SPINACIA OLERACEA.—Fresh leaves of spinach were carefully picked over to remove all imperfect tissue and then ground up and the juice removed by a screw press. The residue was rubbed up with a little water and the fluid again pressed out. The turbid dark green extract was examined as follows:

Flask no. 1—50^{cc} extract—boiled.
Flask no. 2—50^{cc} extract—unboiled.
Flask no. 3—50^{cc} extract + .5 ^{gm} Witte peptone—boiled.
Flask no. 4—50^{cc} extract + .5 ^{gm} Witte peptone—unboiled.
Flask no. 5—50^{cc} extract + .5 ^{gm} Witte peptone + 0.25 ^{gm} citric acid—boiled.
Flask no. 6—50^{cc} extract + .5 ^{gm} Witte peptone + 0.25 ^{gm} citric acid—unboiled.

The antiseptic was chloroform, the length of digestion 30 hours. Ten cubic centimeters of each digestion were tested by acidifying with acetic acid, drying, taking up in alcohol and filtering off the residue, drying the alcoholic extract, taking up the residue in water, and applying the tryptophan test with chlorine water. The results were negative except in the case of no. 4, which showed a fair tryptophan reaction. This result shows that the leaves of spinach contain an enzyme capable of attacking Witte peptone, but not acting on the proteids of the tissue, or if it does these proteids do not yield tryptophan on hydrolysis.

BRASSICA OLERACEA.—The leaves of a half-matured cabbage, some of which were green and some etiolated, were run through a chopping machine and the pulp macerated with water. The fluid was removed by a screw press and the pale green turbid extract filtered. This extract was faintly acid to litmus, but not to lacmoid, and yielded a coagulum on boiling, the filtrate from which gave a faint tryptophan reaction. The extract was examined in the following way:

Flask no. 1—50^{cc} extract.
Flask no. 2—50^{cc} extract—boiled.
Flask no. 3—50^{cc} extract $+ 1^{cc}$ 4 per cent. HCN
Flask no. 4—50^{cc} extract $+ 1^{cc}$ 4 per cent. HCN—boiled.
Flask no. 5—50^{cc} extract $+ 1^{cc}$ 4 per cent. HCN + 0.5^{gm} Witte peptone.
Flask no. 6—50^{cc} extract $+ 1^{cc}$ 4 per cent. HCN + 0.5^{gm} Witte peptone—boiled.
Flask no. 7—50^{cc} extract +0.25 $Na_2 CO_3$.
Flask no. 8—50^{cc} extract +0.25 $Na_2 CO_3$—boiled.

Ten drops of toluol were added to nos. 1, 2, 7, and 8. The flasks were corked and placed in the incubator. After twenty hours' digestion 10^{cc} was removed from each flask, boiled, filtered, and 1^{cc} of acetic acid added to each filtrate. The tryptophan test with chlorine water showed that proteolysis had taken place in all the unboiled digestions except no. 3; that in no. 1 was feeble compared with the action in nos. 5 and 7. These results show that although the enzymes of the cabbage leaves act on the proteids of the tissue, yet that action is slight compared with that exerted upon Witte peptone. The tissue residue from which the extract had been obtained was shaken up with water and toluol, a small portion removed and boiled, and Witte peptone added to both parts. After two days' digestion a sample of the fluid in each flask was tested for tryptophan; the unboiled digestion gave a strong reaction, the boiled one a very faint reaction. The fluid was removed as far as possible from the unboiled digestion mixture, concentrated to a syrup, treated with several volumes of alcohol to remove the proteoses and peptones, and the filtrate concentrated to a thick syrup. After standing, very characteristic crystals of both leucin and tyrosin separated.

DAUCUS CAROTA.—Blossoms of the wild carrot were ground in a mortar with water. The mashed tissue and water were divided into four parts and placed in test tubes.

Tube no. 1—tissue + H_2O + toluol + uncoagulated egg albumen.
Tube no. 2—tissue + H_2O + toluol + uncoagulated egg albumen—boiled.
Tube no. 3—tissue + H_2O + Witte peptone + toluol.
Tube no. 4—tissue + H_2O + Witte peptone + toluol—boiled.

The egg albumen was added because a test of the tissues with

Adamkiewicz reaction, using glyoxylic acid and concentrated sulphuric acid, gave no well-defined positive result. After two days, digestion no. 3 was found to give a weak tryptophan reaction, whereas none of the others gave a positive result. An experiment carried out in the same way with the developing seeds gave the same result except that the unboiled test with Witte peptone gave a much stronger reaction for tryptophan, showing that the proteolysis had been more vigorous. The evidence indicates that the enzyme present is an ereptase.

CASTANEA SATIVA AMERICANA.—The leaves of this tree were ground with sand in a mortar, the pulp mixed with water, divided between four test tubes, and examined as follows:

No. 1—toluol + uncoagulated egg albumen.
No. 2—toluol + uncoagulated egg albumen—boiled
No. 3—toluol + Witte peptone
No. 4—toluol + Witte peptone—boiled.

After two days in the incubator no. 3 gave a faint reaction for tryptophan, the others none. The original pulp from the leaves gave no Adamkiewicz reaction. The unripe seeds of the chestnut were ground to a paste and mixed with water. No Adamkiewicz reaction was given by this mixture, which was divided between four test tubes and examined in precisely the same way as the leaves. The result was qualitatively the same, but the tryptophan reaction in the unboiled trial with Witte peptone was very strong, showing the presence of an active protease, probably an ereptase.

PHASEOLUS MUNGO.—Three weeks old etiolated seedlings of this bean were rubbed up in a mortar and covered with glycerin and a little toluol. After extracting for twenty-two hours, the glycerin was somewhat diluted, strained through gauze, and filtered through absorbent cotton. This extract was tested as follows:

No. 1—10^{cc} extract + fibrin.
No. 2—10^{cc} extract boiled + fibrin.
No. 3—10^{cc} extract + Witte peptone.
No. 4—10^{cc} extract + Witte peptone—boiled.

Examined after three days' digestion no. 1 showed no digestion of the fibrin and gave no test for tryptophan, no. 3 gave a marked tryptophan reaction, and no. 4 none.

CUCURBITA MAXIMA.—The seeds of this plant and the young seedlings with radicles an inch and a half long were found to contain an ereptase of considerable power as shown by the action of their tissues on Witte peptone Under conditions for autolysis none seemed to take place, since no tryptophan reaction was obtainable, notwithstanding the fact that the proteids of this seed give a strong Adamkiewicz reaction. When the seedlings were thirteen days old, however, they were found capable of autolysis with the formation of tryptophan. The results show that the seeds contain an ereptase and that after germination has proceeded for some time a tryptase also makes its appearance.

CUCURBITA PEPO.—The seeds of the pumpkin give the same evidence of the presence of an ereptase as do those of the Hubbard squash. Two attempts were made to obtain preparations of this enzyme. A quantity of ground seeds of the pumpkin were whipped up in water, and the shells, which floated on the surface, were skimmed off. After extracting for several hours, the mixture was strained through gauze and an attempt made to filter it clear. The fluid appeared to have a quantity of emulsified fat in it, and even the repeated filtration through pulp filters failed to make any impression on it. The mixture was allowed to stand two days with toluol to prevent bacterial action and was then readily filtered, yielding a perfectly clear filtrate, which however possessed no proteolytic power. In the second attempt 45^{gm} of ground seeds were extracted with sufficient water to make 275^{cc} of milky looking extract which was found to have a marked action on Witte peptone. As soon as this extract was strained from the ground seeds it was mixed with an equal volume of saturated ammonium sulphate solution and allowed to stand over night. The precipitate was filtered off the next day, and when tested with Witte peptone was found to be very active, showing that the enzyme is either precipitated by half saturation of its solution with ammonium sulphate, or else it sticks to the proteid precipitated by the salt. The precipitate was whipped up in water, transferred to a parchment paper bag, and dialyzed for two days in running water. When taken from the dialyzer and filtered clear, the solution was found to be inactive on Witte peptone. These results show that this enzyme is very sensitive and that some other means must be resorted to in order to obtain preparations of it free from the bulk of proteids, etc., in the tissue extracts.

STUDY OF THE PROTEASES OF PHASEOLUS VULGARIS

The beans used in the experiments to be described were of two sorts, the red kidney bean and the white medium field bean; there has been nothing to suggest that the proteases in these varieties are different, and in the records of experiments no mention will be made of which kind was used.

Resting seeds.—The resting seeds were tested a number of times for proteases; the results always showed that the seeds contained an enzyme capable of hydrolyzing Witte peptone with the formation of tryptophan, but incapable of digesting the phaseolin of the seed. Later in this paper will be found a more detailed description of this ereptase. The record of a typical experiment is given here.

Seeds of *Phaseolus vulgaris* which were known to germinate readily were ground to a fine meal, one gram of this powder placed in each of four test tubes, and further additions made as follows:

No. 1—15^{cc} H_2O+toluol.
No. 2—15^{cc} H_2O+toluol—boiled.
No. 3—15^{cc} H_2O+toluol+0.3^{gm} Witte peptone.
No. 4—15^{cc} H_2O+toluol+0.3^{gm} Witte peptone—boiled.

All were corked, shaken, and kept in the incubator at 37–39° C. for forty-one hours. At the expiration of that time the contents of each tube were boiled, acidified with acetic acid, and filtered. The filtrates were then tested.

No. 1—no biuret, Millon's, nor tryptophan reactions.
No. 2—not tested.
No. 3—strong tryptophan reaction.
No. 4—no tryptophan reaction.

Seeds soaked in water for twenty-four hours.—Seeds from the same lot used in the above experiment were soaked in distilled water for twenty-four hours, the hulls removed, and the cotyledons ground in a mortar and 1.5^{gm} of the paste tested in the same way as the dry bean meal, and with the same results.

Seeds soaked for forty-five hours.—The beans not used in the last experiment were soaked twenty-one hours longer, peeled, and the tests repeated, with the same results.

Seeds soaked twenty-four hours and germinated in sand three days. —At this stage the hypocotyls were about one inch long. The cotyledons were removed, ground in a mortar, and the paste tested as in the previous experiments. The result was the same, the Witte peptone was attacked and the seed proteids were not.

Seeds soaked twenty-four hours and germinated six days.—The cotyledons from the young plants with hypocotyls two to three inches long were removed and ground to a paste. One gram of this was placed in each of six test tubes and other additions made as follows:

No. 1—10^{cc} H_2O + toluol.
No. 2—10^{cc} H_2O + toluol—boiled.
No. 3—10^{cc} H_2O + 0.1 per cent. of citric acid + toluol.
No. 4—10^{cc} H_2O + 0.1 per cent. of Na_2CO_3 + toluol.
No. 5—10^{cc} H_2O + 0.2^{gm} Witte peptone + toluol.
No. 6—10^{cc} H_2O + 0.2^{gm} Witte peptone—boiled + toluol.

After nineteen hours in the incubator the contents of each tube were boiled, acidified, filtered, and the filtrates tested with these results:

No. 1—no tryptophan nor biuret reactions, Millon's?
No. 2—not tested.
No. 3—no tryptophan reaction, biuret? Millon's?
No. 4—no tryptophan reaction, biuret? Millon's?
No. 5—strong tryptophan reaction.
No. 6—no tryptophan reaction.

Seeds soaked twenty-four hours and germinated ten days in the dark. —The cotyledons at this age of the plant were comminuted and tested in tubes as follows:

No. 1—ground cotyledons + H_2O + toluol.
No. 2—ground cotyledons + H_2O + toluol + uncoagulated egg albumen.
No. 3—ground cotyledons + H_2O + toluol + edestin.
No. 4—ground cotyledons + H_2O + toluol + Witte peptone.

After eighteen hours in the incubator the tests showed that only the Witte peptone had undergone proteolysis.

Cotyledons of eleven-day old seedlings grown in light.—Beans allowed to germinate in washed sand, with a good supply of light, for eleven days had attained a height of five to six inches and had two well developed leaves besides the shriveled cotyledons. The cotyledons were found still to contain an active ereptase.

Cotyledons of etiolated seedlings thirteen days old.—These cotyledons gave convincing evidence of the presence of ereptase, but no tryptase.

Besides testing the cotyledons of germinating beans by the method of autodigestion, extracts were also prepared with glycerin and 20 per cent. alcohol. The tests applied to these extracts showed that they were incapable of digesting the bean proteids or boiled fibrin.

It should be noted that OSBORNE and HARRIS[17] have found that phaseolin, the globulin which makes up the main proteid store of the resting bean, gives but a faint Adamkiewicz reaction. Therefore the absence of a tryptophan reaction in the autodigestion experiments with the bean cotyledons would not be conclusive evidence of the lack of proteolysis. The use of the Millon's reaction for tyrosin and the biuret reaction for albumoses and peptones would have shown the presence of the products of peptonization or proteolysis had they been present. Still more conclusive evidence that the enzyme in question does not act on the unaltered proteids of the cotyledons is afforded by the more thorough study of the enzyme.

Beans were allowed to germinate for four days and the cotyledons separated and ground in a mortar to a paste. Forty grams of this pulp were extracted with 200^{cc} of water and 5^{cc} of toluol. The extract was removed from the tissue residue by straining through fine muslin, and, since phaseolin is soluble in water and the salts of the seed, this milky looking fluid was rich in proteid matter. The 200^{cc} of extract were divided between two flasks and one of the portions boiled. After digesting for seventeen and one-half hours, a comparative quantitative estimation of the amount of nitrogen in the proteid decomposition products was made in the following manner. Fifteen cubic centimeters of each digestion fluid were mixed in a dry beaker with 15^{cc} of tannic acid reagent (7 per cent. of tannic acid dissolved in 2 per cent. acetic acid) and the precipitates removed by filtration. Nitrogen determinations were made in duplicate by the Kjeldahl method on 5^{cc} of each filtrate. The amount of standard acid required to neutralize the ammonia formed by the sulphuric acid treatment was, in the unboiled digestion, 0.7^{cc}; in the boiled digestion 0.5^{cc}. The digestions were allowed to proceed for two days longer and the tests repeated. This time the unboiled digestion required 0.5^{cc} of acid; the boiled digestion required 0.5^{cc}.

---

[17] OSBORNE and HARRIS, *loc. cit.*

This experiment was repeated, using different conditions of reaction to make sure that such conditions played no part in the results previously obtained. Fifty grams of cotyledons from seven-day etiolated bean seedlings were comminuted and an extract prepared as before. This was divided between five flasks as follows:

No. 1—50^{cc} extract.
No. 2—50^{cc} extract—boiled.
No. 3—50^{cc} extract + 0.14^{gm} citric acid ($=\frac{N}{25}$ acid).
No. 4—50^{cc} extract + 0.106^{gm} Na_2CO_3 ($=\frac{N}{25}Na_2CO_3$).
No. 5—50^{cc} extract + 2^{gm} Witte peptone.

Toluol was added in equal amount to all the flasks. The quantitative estimations were made in the same manner as in the last experiment except that 10^{cc} of each filtrate were used for an analysis instead of 5^{cc}. In place of a boiled control for no. 5, a determination was made at the start of the experiment, when it appeared that 1.4^{cc} of standard acid were required to neutralize the ammonia formed from the nitrogen compounds in 10^{cc} of the filtrate from the tannic acid precipitate. After three days' digestion quantitative determinations were made on all.

No. 1—required 1.3^{cc} of standard acid.
No. 2—required 1.0^{cc} of standard acid.
No. 3—required 1.5^{cc} of standard acid.
No. 4—} analyses lost by breaking of the apparatus.
No. 5—}

Three days later the tests were repeated.

No. 1—required 1.4^{cc} standard acid.
No. 2—analyzed 5^{cc} of digestion fluid = to 10^{cc} of tannic acid filtrate; required 4.3^{cc} standard acid.
No. 3—required 1.4^{cc} standard acid.
No. 4—required 1.1^{cc} standard acid.
No. 5—required 6.5^{cc} standard acid.

The digestion fluid of no. 5 at the close of the experiment gave a strong tryptophan reaction. The variations among the digestions, except no. 5, are within 0.5^{cc} of standard acid; in such a method these differences cannot be held to show the presence of a trypsin-like enzyme, especially when we see the change in the digestion containing Witte peptone which at first required 1.4^{cc} of acid for 10^{cc} of filtrate and at the close 6.5^{cc}.

Being convinced by the experiments tried that the resting and germinating bean contained an ereptase, I attempted to separate it from the mass of other materials in the seed extracts.

One hundred and fifty grams of bean meal were extracted for five hours with 375^{cc} of water with the addition of toluol and chloroform. The fluid was strained and pressed out through cheese cloth, the residue mixed with 150^{cc} of water and this likewise removed. The total extract, amounting to 450^{cc}, was allowed to settle over night to get rid of the starch, etc. by sedimentation. The next morning the supernatant fluid was siphoned off and filtered three times through pulp filters, yielding 365^{cc} of an opalescent fluid. This was half saturated with ammonium sulphate by adding an equal volume of saturated ammonium sulphate solution. The flocculent precipitate *A* was removed by filtration, and to the filtrate saturated ammonium sulphate solution was added to make two-thirds saturation. Precipitate *B* was likewise removed and ammonium sulphate added to three-quarters saturation. With this concentration of the salt nothing but a turbidity resulted; accordingly ammonium sulphate in substance was added to saturation, yielding the precipitate *C*. A small portion of each precipitate was placed in a test tube and mixed with Witte peptone and water; after standing over night in the incubator the fluids were tested for tryptophan. The results showed that *A* contained sufficient ereptase to cause a strong tryptophan reaction, *B* a weak one, and *C* none at all. Each precipitate was dissolved in water so far as possible, and the turbid solution placed in dialyzing bags and dialyzed until practically free from sulphates. In *A* a considerable amount of phaseolin was precipitated on the sides of the dialysis; this was filtered off, washed with water, and both the clear filtrate and the globulin tested with Witte peptone. It was found that the ereptase had remained in solution and had not been carried down with the precipitated proteid. The solutions of the precipitates *B* and *C*, after dialysis, were found to have respectively a weak action on Witte peptone and no action at all.

The ereptase solution obtained from *A* gave a weak biuret reaction and a faint coagulum on heating. Attempts to precipitate the enzyme by means of uranium acetate according to Jacoby's method failed; no precipitate was obtained except in acid solutions in which the enzyme was destroyed. The ereptase was tested with crystallized edestin from hemp seed, crystallized excelsin from the Brazil nut, phaseolin from the bean, boiled fibrin, and Witte peptone. The native proteids were all unattacked and the Witte peptone was actively digested.

An ereptase solution was prepared from 1500^{gm} of bean meal by extraction with water, filtering the extract, half saturating the solution with ammonium sulphate, and dialyzing the solution obtained from this half saturation precipitate. A perfectly clear solution resulted after filtering off the phaseolin precipitated by dialysis, and this was dried at 40 to 50° C., yielding 2.9^{gm} of a golden yellow powder. This powder was found to be active in digesting Witte peptone, although it seemed that the purification and drying had considerably diminished the power of the enzyme. This protease preparation was tested with some of the fractions of Witte peptone which had been separated by PICK'S[18] method and with a preparation of phaseolin. The first experiment was carried out using the secondary protease preparations without the removal of the ammonium sulphate left adherent by the method of separation.

One and one-half grams of the enzyme preparation mentioned above were dissolved as completely as possible in 300^{cc} of water, 35^{cc} of this solution measured into each of five flasks, and 2^{cc} of toluol added. Proteids were added as follows:

No. 1—1^{gm} of phaseolin.
No. 2—1^{gm} of proto-proteose.
No. 3—1^{gm} of hetero-proteose.
No. 4—1^{gm} of secondary albumose *A*.
No. 5—1^{gm} of secondary albumose *B*.

After shaking, 15^{cc} were removed from each flask, 15^{cc} of tannic acid reagent added, and the precipitates filtered off. Analyses of 10^{cc} of each filtrate were made in duplicate:

No. 1—required 0.7^{cc} of standard acid.
No. 2—required 0.6^{cc} of standard acid.

[18] PICK, Zeitschr. Physiol.-Chem. **24**: 246. 1898.

No. 3—required 0.8^{cc} of standard acid.
No. 4—required 10.3^{cc} of standard acid.
No. 5—required 7.6^{cc} of standard acid.[19]

After six days in the incubator the quantitative estimations were repeated.

No. 1—required 0.6^{cc} standard acid = gain of 0.0^{cc}.
No. 2—required 1.9^{cc} standard acid = gain of 1.3^{cc}.
No. 3—required 1.3^{cc} standard acid = gain of 0.5^{cc}.
No. 4—required 10.9^{cc} standard acid = gain of 0.6^{cc}.
No. 5—required 9.2^{cc} standard acid = gain of 1.6^{cc}.

The results in the case of the phaseolin, the proto-proteose, and the deutero-albumose *B* are well defined; those with the hetero-albumose and the deutero-albumose *A* were not sufficiently marked to lead to a definite conclusion.

A second experiment was tried with the four proteose preparations after the ammonium sulphate had been removed from the two deutero-proteoses. In these trials, instead of using the ereptase preparation used in the previous experiment, an extract was made from some bean meal and filtered several times through pulp filters until it was nearly clear. Of each of the four proteoses 0.125^{gm} was dissolved in 25^{cc} of water. Ten cubic centimeters of the bean extract were placed in each of eight test tubes and four of the portions boiled. Ten cubic centimeters of each proteose solution were added to a boiled, and 10^{cc} to an unboiled, portion of the extract. Nine drops of toluol were added to each tube, the tubes corked, shaken, and kept in the incubator for sixty-five hours. At the expiration of that time the contents of all the test tubes were boiled and filtered. Of each filtrate 10^{cc} were removed to a clean test tube, 5^{cc} of a 10 per cent. sodium hydroxide solution added, and then dilute copper sulphate to the maximum biuret reaction. After standing for several minutes the strengths of the biuret reactions from the boiled and unboiled digestions of the different proteoses were compared. The reactions showed that all of the proteoses were attacked by the enzyme. The portions of the filtrates not used in the comparative biuret reactions were employed to test for tryptophan. The unboiled digestions with proto-proteose, hetero-proteose, and deutero-proteose *A* gave distinct tryptophan tests, that with deutero-proteose *B* was very faint; none of the boiled digestions showed any trace of tryptophan.

---

[19] The ammonium sulphate in the last two proteose preparations accounts for the high nitrogen in the filtrates from the tannic acid precipitates.

In summing up the results of the experiments described above, we may note that evidence of the presence of a protease has been obtained in the leaves of the spinach and cabbage, the blossoming heads of *Daucus Carota* and the developing seeds of that plant, in the leaves and unripe seeds of the chestnut, the etiolated seedlings of *Phaseolus Mungo*, the seeds and seedlings of *Cucurbita maxima*, and the seeds of *Cucurbita Pepo*. In the case of *Phaseolus vulgaris*, experiments with all stages of the germination of the seed have shown that the cotyledons always contain an enzyme of the erepsin group, and at no time can any evidence of the presence of an enzyme capable of attacking the proteids of the seed be obtained. This ereptase may be removed from the bean extracts by half saturation of the solution with ammonium sulphate. On dissolving this precipitate in water and dialyzing the solution free from sulphates, the enzyme is obtained in a solution which is perfectly clear and gives but feeble reactions for proteids. A preparation of the enzyme may be obtained by drying this solution at a temperature below 50° C. The enzyme acts on the proto-proteose, the hetero-proteose, and the deutero-proteoses, separated from Witte peptone; it is quite inactive on phaseolin of the bean, excelsin of the Brazil nut, edestin of the hemp seed, and boiled fibrin.

There can be no doubt but that the large proteid store in bean seeds is utilized in germination, and in all probability this utilization is preceded by cleavage to the amido-acids, hexon bases, etc. What effects this cleavage, and what part, if any, the ereptase plays, are unsolved problems. It is likewise unknown whether or not this enzyme occurs in other parts of the plant or whether enzymes of the trypsin type are present in certain tissues.

The investigation of which this is a partial report was carried out with the assistance of a grant from the Carnegie Institution.

Laboratory of Plant Physiology,
Sheffield Scientific School,
Yale University